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14. ABSTRACT: Protein phosphorylation plays a key role in the regulation of the function of the proteins and the control of wild range of cellular process. Odin, one of signaling molecules identified in EGF receptor signaling pathway, functions as a negative regulator of growth factor receptor signaling pathways. To dissect the molecular mechanism of Odin in signaling pathway and its biological function, we employed SILAC methodology to identify 36 interacting proteins of Odin, 34 of which are novel. In the future, we seek to verify these interactions by in vitro and in vivo experiments. Since several interacting proteins of Odin play an important role in downregulation of RTK by endocytosis, we also want to examine the function of Odin and its phosphorylation in this process.

15. SUBJECT TERMS
Odin, Peptide array, phosphorylation, SILAC, mass spectrometry

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Introduction

Tyrosine kinase mediated signaling events are important for controlling a diverse range of cellular processes ranging from proliferation and cell migration to apoptosis. Dysregulated tyrosine kinase signaling is responsible for a large number of cancers, and several tyrosine kinases are being actively pursued as therapeutic targets for their treatment. Using a mass spectrometry-based proteomic approach, we have previously identified a number of signaling molecules in the EGF receptor (EGFR) signaling pathway. One of the novel molecules, designated Odin, acts as an inhibitor of growth factor receptor signaling pathways. The objective of our work is to investigate the molecular basis of its inhibitory activity, and to establish its role in transformation induced by tyrosine kinases and in mammary development and tumorigenesis.

Body

During last year, the following progress has been made to characterize the role of Odin in growth factor receptor signaling.

To identify proteins associated with Odin using the SILAC methodolody.

Firstly, we have examined the tyrosine phosphorylation of Odin in 293T human embryonic kidney cells under the stimulation of growth factor. FLAG-tagged Odin were overexpressed with wild type or kinase dead(K745M) EGF receptor in 293T cells using Lipofectamine 2000 transient transfection kit (Invitrogen) and subject to immunoprecipitation using anti-FLAG antibodies (Sigma). The tyrosine phosphorylation of Odin in the immunoprecipitates were examined by western blotting with anti-phosphotyrosine antibodies (4G10, Upstate) and the amout of overexpressed Odin and EGF receptor were examined by reprobing the membrane with anti-FLAG antibodies. In consistent with the previous report (*1*), Odin was phosphorylated by overexpressed EGF receptor while the expression of Odin and EGF receptor are similar (Figure 1).

In order to identify proteins associated with Odin, we performed a large-scale experiment using SILAC methodology, shown in Figure 2. Two sets of 293T cells were grown in the media containing either light isotopic ($^{12}C_6$-Lys/Arg) or heavy isotopic ($^{13}C_6$-Lys/Arg) amino acids. The set growing in light media were transfected with human EGF receptor and empty vector (pCMV-Tag4A) while the set growing in heavy media were transfected with human EGF receptor and FLAG tagged Odin in pCMV-Tag4A using Lipofectamine 2000 transient transfection kit under manufactary manual (Invitrogen). After 24 hours transfection, both sets of 293T cells were starved for 18 hours in serum-free DMEM and lyzed in coimmunoprecipitation buffer. The protein concentration of the lysates from both sets of cells were measured by Lowry method. Both lysates with the same amount of proteins were precleared using Protein G-agrose beads (Sigma), filtered with 0.45μm sterile filter and incubated with anti-FLAG M2® agrose beads for 6 hours, separately. After washing, the immunoprecipitates from each transfection were mixed and the bound proteins were eluted using 1 mg/ml FLAG peptides (Sigma). The eluates were dialyzed, concentrated and resolved on 10% SDS-PAGE. After colloidal Coomassie staining, the gel was excised into 36 bands and the proteins in each band were digested with trypsin using in-gel digestion protocols. After tryptic digestion, the resulting peptides were extracted and analyzed by reversed phase liquid chromatography tandem mass spectrometry (LC-MS/MS). Briefly, the peptides were injected into in-house prepared trap packed with C_{18} particles (ODS-A YMC), using an Agilent 1100 autosampler (Agilent Technologies, Palo Alto, CA). Peptides were eluted from the trap onto an analytical C_{18} column using a 5-60% acetonitrile gradient. The data will be acquired on a Micromass Q-TOF US-API mass spectrometer (Waters, Milford, MA) or a Q-

STAR Pulsar mass spectrometer (Applied Biosystems/MDS Sciex, Foster City, CA) and analyzed by MASCOT (Matrix Science, Boston) using the RefSeq database downloaded from National Center for Biotechnology Information. The relative quantitation of identified proteins was obtained using MSQuant (http://msquant.sourceforge.net/).

Identification of Proteins associated with Odin

Totally, more than 250 proteins were identified from our analysis. Relative quantitation analysis divides all proteins into four categories:

1) Proteins only have heavy amino acid labeled peptides. FLAG-tagged Odin belongs to this category and all peptides from Odin are heavy peptides (Table 1). The MS spectra of three representative peptides from Odin are shown in Figure 3. Additionally, the two known phosphorylation site on Odin (Ser647/Ser663)(*2, 3*) were also detected (Table 2), while the corresponding non-phosphorylated peptides are also identified. This category also includes interacting proteins of Odin (Table 2). The MS spectra of one representative peptide from one protein in this category, CD2 associated protein, are shown in Figure 4A.

2) Proteins have both heavy and light amino acid labeled peptides and the ratio of heavy over light peptides from these proteins is more than 2.5 (Table 2). The proteins from this category are also the interacting proteins but they also bind antibodies nonspecifically. The MS spectra of one representative peptide from one protein in this category, peroxiredoxin 1, are shown in Figure 4C.

3). Proteins have both heavy and light amino acid labeled peptides and the ratio of heavy over light peptides from these proteins is less than 2.5 and more than 0.4 (data not shown). These proteins are contaminating proteins which bind to antibodies and agrose beads non-specifically. The MS spectra of one representative peptide from one protein in this category, ribosomal protein S7, are shown in Figure 4C.

4). Proteins only have light amino acid labeled peptides. These proteins are contaminating proteins from outside and most are keratins.

Bioinformatic analysis of interacting proteins of Odin in category one

Although Odin has been identified in the protein complex in 14-3-3 gamma and sigma (*4, 5*), our data indicated that all seven members of 14-3-3 adaptor protein family form the protein complex with Odin. One known phosphorylation site Ser647 is one of eight candidate 14-3-3 protein binding sites predicted by Phosphomotif Finder (*6*). Three interacting proteins of Odin, RASAL2, DAB2IP and ARHGAP10, are belong to the GTPase activating proteins family, of which RASAL2 and ARHGAP10 were reported as interacting proteins of 14-3-3 proteins (*5, 7*). DAB2IP has also been proposed to play an inhibitory role in the growth of prostate cancer (*8*). CD2AP and SH3KBP1 both contain three SH3 domains and have been proposed to play an important role in the downregulation of RTK (*9*). Both proteins have been found to bind CAPZ (*10*), while CAPZB is also identified as interacting protein of Odin in our analysis. Additionally, another interacting protein of Odin, VAPA, is proposed to be involved in vesicle fusion (*11*). Talin2 is a cytoskeleton binding protein, while UACA is an adaptor protein based on the analysis of domain structure. GART is one metabolic enzyme while HSPA9B belongs to the heat shock protein 70 family.

Key Research Accomplishments

1. We have identified 36 interacting proteins of Odin, 34 of which are novel. The preliminary analysis of these proteins showed the success of SILAC in distinguishing true interacting protein of Odin from contaminating protein. These analysis gave the hint about the function of Odin.

Future Plans:

Our major goal over the next year is to verify the interactions by systematic in vitro and in vivo experiments using GST and epitope-tagged constructs to determine whether the binding is direct and the exact regions of interaction. We also want to study the functions of Odin and its interactions with its partners in the regulation of receptor tyrosine kinase signaling. Since several interacting proteins of Odin have been shown to be involved in negative regulation of receptor tyosine kinase by promoting endocytosis of activated receptors, it is obvious to study the functions of Odin in this process, especially whether the endocytosis of activated receptors are affected in Odin knock out mouse embryonic fibrablasts.

Publications: None.

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APPENDICES: None

SUPPORTING DATA:

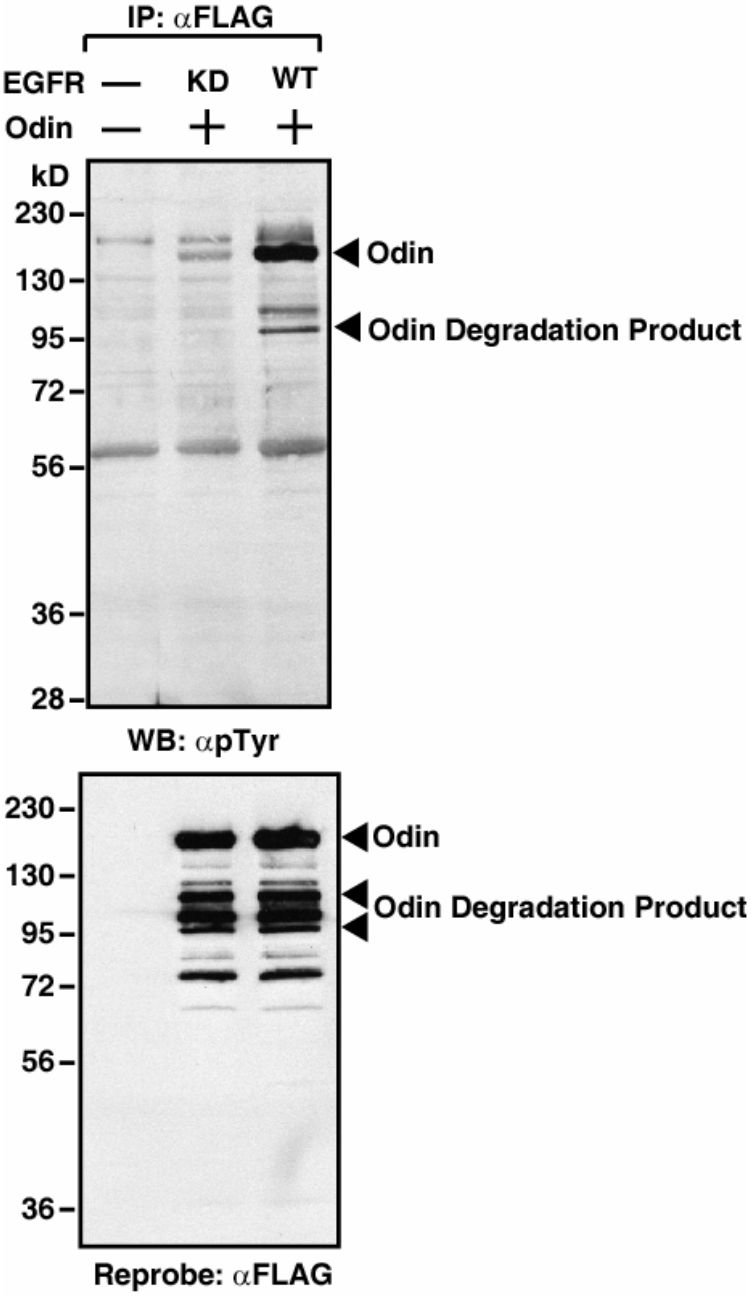

Figure 1. Exogenous Odin are tyrosine phosphorylated upon activated EGF receptor. Three 10 cm dishes of 293T cells are transiently transfected with vector control or kinase-dead (KD) EGFR/FLAG-tagged Odin or wild type (WT) EGFR/FLAG-tagged Odin, separately. After 24 hours transfection, 293T cells are starved for 8 hours, harvested in co-immunoprecipitation buffer and immunoprecipitated by anti-FLAG antibodies. The immunoprecipitates are resolved on SDS-PAGE and the tyrosine phosphorylation of Odin are detected by western blotting with anti-phosphotyrosine antibodies (4G10, Upstate) and the expression of Odin are detected by reprobing the membrane with anti-FLAG antibodies (Sigma).

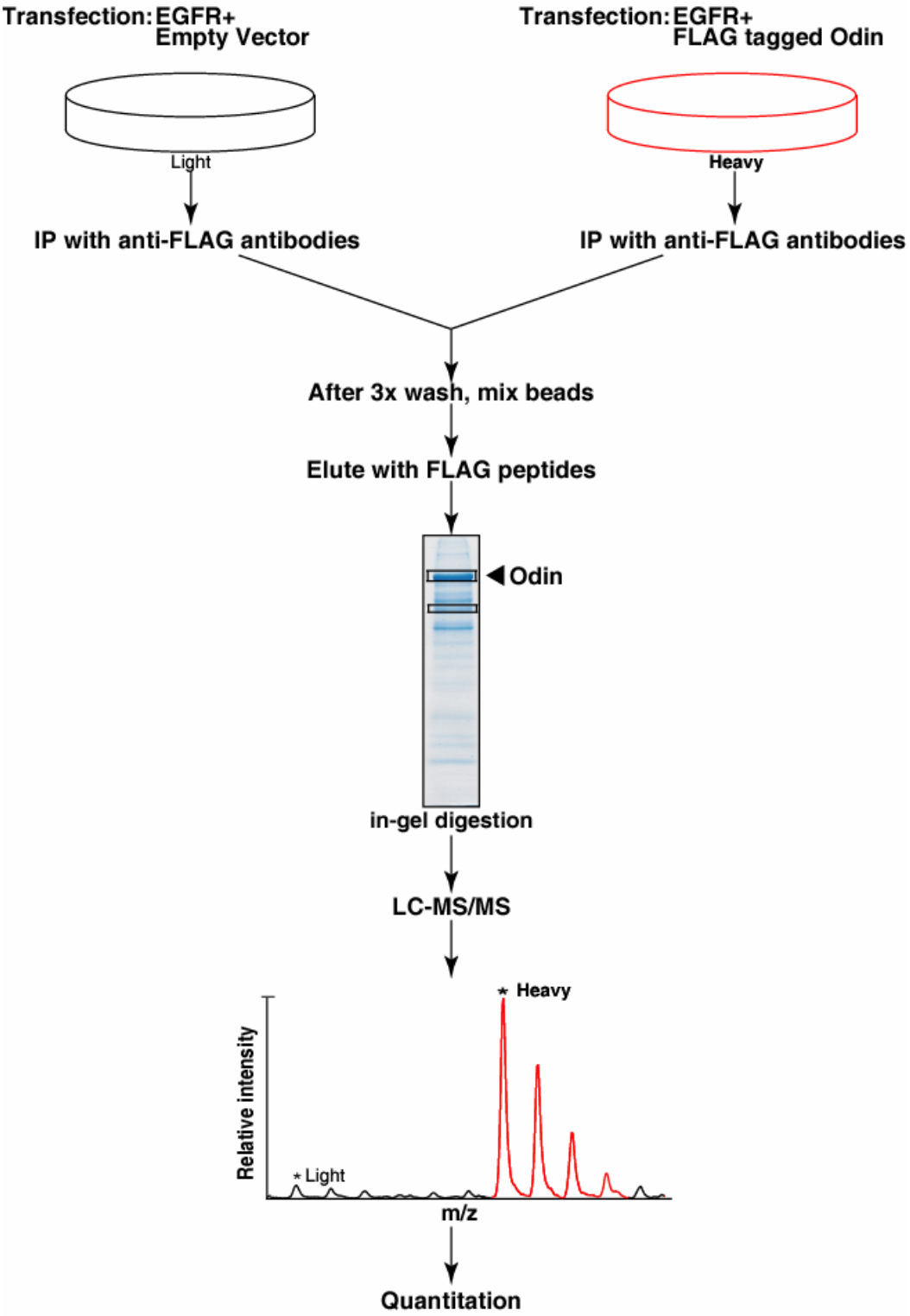

Figure 2. Schematic of the SILAC methodology to identify proteins associated with Odin. Two sets of 293T cells were grown in either light (^{12}C) or heavy (^{13}C) amino acids containing media as indicated. Cells grown in light media were transfected with human EGFR and empty vector as control while cells grown in heavy media were transfected with human EGFR and FLAG-tagged Odin. After starvation, both sets of cells were lyzed and immunoprecipitated using anti-FLAG antibodies. After washing, the immunoprecipitates were mixed and the bound proteins were eluted with FLAG peptides. The proteins were resolved by SDS-PAGE. The gel was stained and the protein bands excised, digested with trypsin, and analyzed by LC-MS/MS. The absence of light MS ion peak will indicate the true interacting protein of Odin.

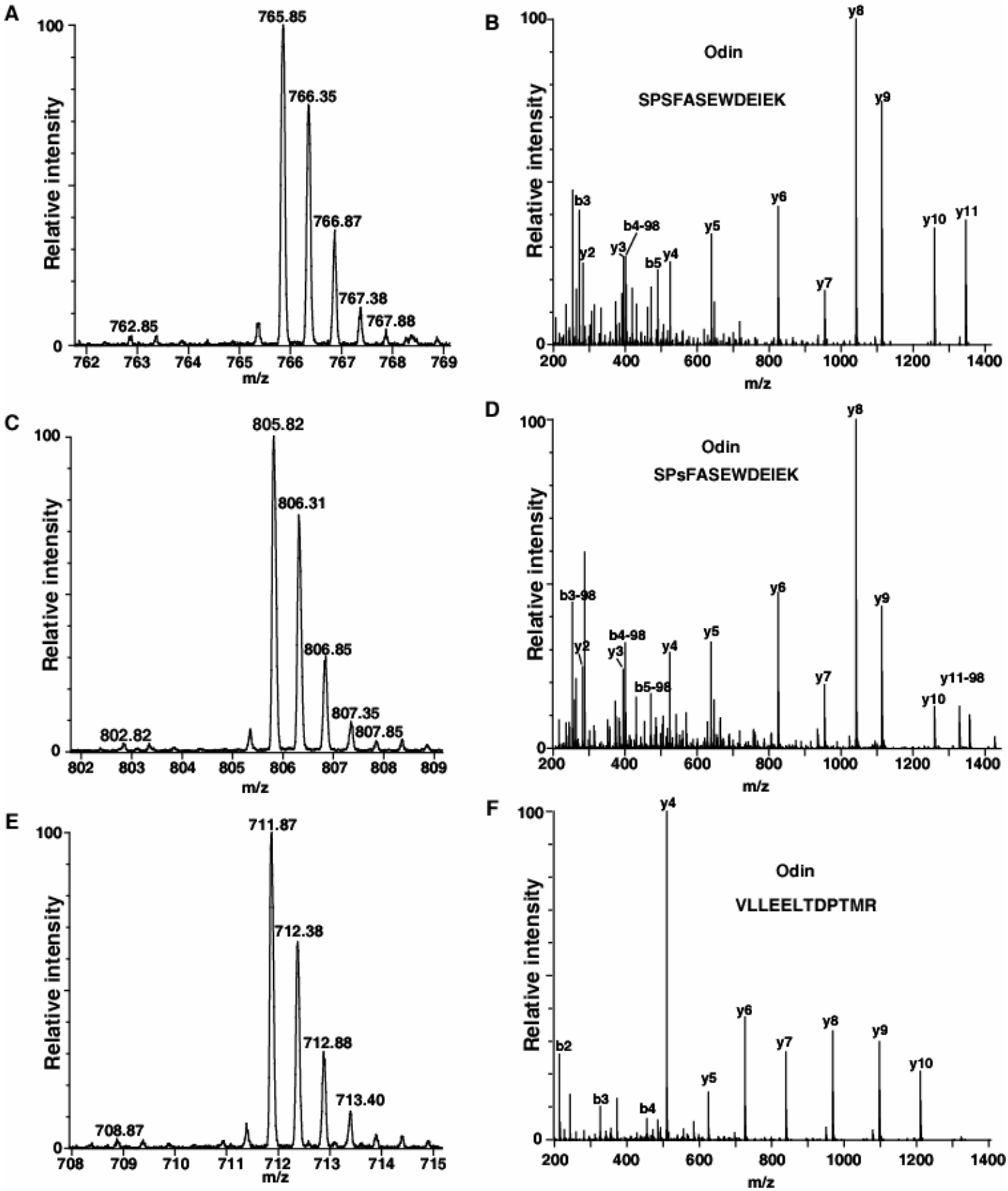

Figure 3. Odin is completely labeled with heavy isotope amino acids. MS spectra of three representative peptides from Odin are shown on the left. The nearly absence of light ion peak indicated that Odin are fully labeled with heavy isotope. The panels on the right show the corresponding MS/MS spectra along with the peptide sequence.

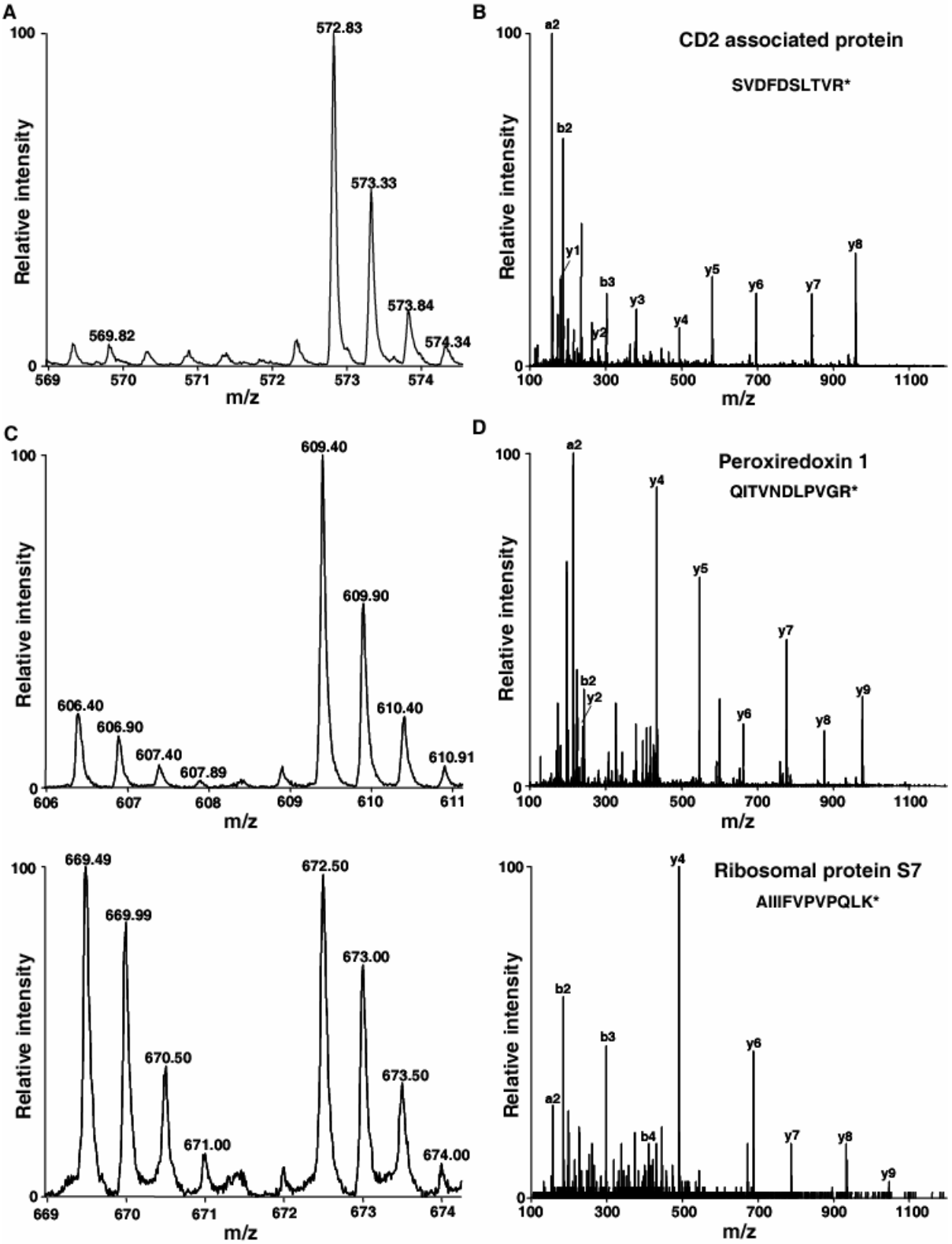

Figure 4. Three different groups of proteins are identified. MS spectra of one representative peptide each from CD2 associated protein, peroxiredoxin 1 and ribosomal protein S7 are shown on the left. The panels on the right show the corresponding MS/MS spectra along with the peptide sequence.

Table 1. List of identified peptides from Odin

PEPTIDE SEQUENCE	MODIFICATIONS
K.HTPLHLAAR.N	9:Arginine-13C6 (R-13C6)
K.EQELLEAAR.T	9:Arginine-13C6 (R-13C6)
K.NVIAEHEIR.N	9:Arginine-13C6 (R-13C6)
K.VVLVDGK.T	7:Lysine-13C6 (K-13C6)
R.NDALTNVADSK.G	11:Lysine-13C6 (K-13C6)
R.KHTPLHLAAR.N	1:Lysine-13C6 (K-13C6); 10:Arginine-13C6 (R-13C6)
R.GTESTQDACAK.M	9:Carbamidomethyl (C); 11:Lysine-13C6 (K-13C6)
R.SESLSNCSIGK.K	7:Carbamidomethyl (C); 11:Lysine-13C6 (K-13C6)
R.LLIHQGPSHTR.V	11:Arginine-13C6 (R-13C6)
K.GCYPLHLAAWK.G	11:Lysine-13C6 (K-13C6)
R.SESLSNCSIGK.K	3:Phospho (ST); 7:Carbamidomethyl (C); 11:Lysine-13C6 (K-13C6)
K.SQGDVEK.A	7:Lysine-13C6 (K-13C6)
K.IPTIILSITYK.G	11:Lysine-13C6 (K-13C6)
R.ATGASAAEMIETK.S	13:Lysine-13C6 (K-13C6)
R.ATGASAAEMIETK.S	9:Oxidation (M); 13:Lysine-13C6 (K-13C6)
R.HDSLHDPAAPSR.A	12:Arginine-13C6 (R-13C6)
K.DNHGLTALDTVR.E	12:Arginine-13C6 (R-13C6)
R.SADLLLPPGDTGR.R	13:Arginine-13C6 (R-13C6)
K.GCYPLHLAAWK.G	2:Carbamidomethyl (C); 11:Lysine-13C6 (K-13C6)
R.SESLSNCSIGKK.R	7:Carbamidomethyl (C); 11:Lysine-13C6 (K-13C6); 12:Lysine-13C6 (K-13C6)
R.VGYLTGLPTTNSR.S	
R.VGYLTGLPTTNSR.S	13:Arginine-13C6 (R-13C6)
R.SESLSNCSIGKK.R	3:Phospho (ST); 7:Carbamidomethyl (C); 11:Lysine-13C6 (K-13C6); 12:Lysine-13C6 (K-13C6)
K.VLLEELTDPTMR.N	12:Arginine-13C6 (R-13C6)
K.VLLEELTDPTMR.N	11:Oxidation (M); 12:Arginine-13C6 (R-13C6)
K.VLLEELTDPTMR.N	11:Oxidation (M); 12:Arginine-13C6 (R-13C6)
K.FETPLDLAALYGR.L	13:Arginine-13C6 (R-13C6)
K.FETPLDLAALYGR.L	13:Arginine-13C6 (R-13C6)
K.NLWELELVNVLK.V	12:Lysine-13C6 (K-13C6)
R.SADLLLPPGDTGRR.R	13:Arginine-13C6 (R-13C6); 14:Arginine-13C6 (R-13C6)
K.SPSFASEWDEIEK.I	13:Lysine-13C6 (K-13C6)
K.RVGYLTGLPTTNSR.S	1:Arginine-13C6 (R-13C6); 14:Arginine-13C6 (R-13C6)
K.SPSFASEWDEIEK.I	3:Phospho (ST); 13:Lysine-13C6 (K-13C6)
K.FIDASNK.N	7:Lysine-13C6 (K-13C6)
K.SQQIAALIEDHMTGK.R	15:Lysine-13C6 (K-13C6)
K.SQQIAALIEDHMTGK.R	12:Oxidation (M); 15:Lysine-13C6 (K-13C6)
K.IMSSIGEGIDFSQER.Q	15:Arginine-13C6 (R-13C6)
K.IMSSIGEGIDFSQER.Q	2:Oxidation (M); 15:Arginine-13C6 (R-13C6)

K.MLLNAHPNLLSCNTK.K	12:Carbamidomethyl (C); 15:Lysine-13C6 (K-13C6)
K.MLLNAHPNLLSCNTK.K	1:Oxidation (M); 12:Carbamidomethyl (C); 15:Lysine-13C6 (K-13C6)
K.TDVVQILLAAGTDVNIK.D	17:Lysine-13C6 (K-13C6)
K.KVVLVDGK.T	1:Lysine-13C6 (K-13C6); 8:Lysine-13C6 (K-13C6)
K.YFPLTASEVLSMRPR.I	13:Arginine-13C6 (R-13C6); 15:Arginine-13C6 (R-13C6)
K.SQQIAALIEDHMTGKR.S	12:Oxidation (M); 15:Lysine-13C6 (K-13C6); 16:Arginine-13C6 (R-13C6)
R.NNKFETPLDLAALYGR.L	16:Arginine-13C6 (R-13C6)
R.NNKFETPLDLAALYGR.L	3:Lysine-13C6 (K-13C6); 16:Arginine-13C6 (R-13C6)
K.MLLNAHPNLLSCNTKK.H	12:Carbamidomethyl (C); 15:Lysine-13C6 (K-13C6); 16:Lysine-13C6 (K-13C6)
K.FIDASNKNVIAEHEIR.N	7:Lysine-13C6 (K-13C6); 16:Arginine-13C6 (R-13C6)
K.MLLNAHPNLLSCNTKK.H	1:Oxidation (M); 12:Carbamidomethyl (C); 15:Lysine-13C6 (K-13C6); 16:Lysine-13C6 (K-13C6)
R.EEDEHPYELLLTAETK.K	16:Lysine-13C6 (K-13C6)
K.TPPPQPPLISSMDSISQK.S	18:Lysine-13C6 (K-13C6)
K.TPPPQPPLISSMDSISQK.S	12:Oxidation (M); 18:Lysine-13C6 (K-13C6)
R.EEDEHPYELLLTAETKK.V	16:Lysine-13C6 (K-13C6); 17:Lysine-13C6 (K-13C6)
R.IIASLADRPYEEPPQKPPR.F	8:Arginine-13C6 (R-13C6); 16:Lysine-13C6 (K-13C6); 19:Arginine-13C6 (R-13C6)
K.LIFESCGYEANYLGSMLIK.D	6:Carbamidomethyl (C); 19:Lysine-13C6 (K-13C6)
K.LIFESCGYEANYLGSMLIK.D	6:Carbamidomethyl (C); 16:Oxidation (M); 19:Lysine-13C6 (K-13C6)
R.NISCAAQDPEDLCTFAYITK.D	4:Carbamidomethyl (C); 13:Carbamidomethyl (C); 20:Lysine-13C6 (K-13C6)
R.CQDLLSQTSSPLSQNDSCTGR.S	1:Carbamidomethyl (C); 18:Carbamidomethyl (C); 21:Arginine-13C6 (R-13C6)
K.EVDKTPPPQPPLISSMDSISQK.S	4:Lysine-13C6 (K-13C6); 22:Lysine-13C6 (K-13C6)
K.EVDKTPPPQPPLISSMDSISQK.S	4:Lysine-13C6 (K-13C6); 16:Oxidation (M); 22:Lysine-13C6 (K-13C6)
R.TGHLPAVEK.L	9:Lysine-13C6 (K-13C6)
R.SSDQDSTNKEAEAAGVKPAGVRPR.E	9:Lysine-13C6 (K-13C6); 17:Lysine-13C6 (K-13C6); 22:Arginine-13C6 (R-13C6); 24:Arginine-13C6 (R-13C6)
K.ASMQLEETGVHAPGASQPSALDQSKR.V	3:Oxidation (M); 25:Lysine-13C6 (K-13C6); 26:Arginine-13C6 (R-13C6)
K.LTLRPPSLAAPYAPVQSWQHQPEK.L	4:Arginine-13C6 (R-13C6); 24:Lysine-13C6 (K-13C6)
R.VNEQNNDNETALHCAAQYGHTEVVK.V	14:Carbamidomethyl (C); 25:Lysine-13C6 (K-13C6)

Table 2. Interacting proteins of Odin only have heavy amino acid labeled peptides.

GENE SYMBOL	Protein Name(Alternative Name)	The ratio Odin/Control transfection
YWHAG	tyrosine 3-monooxygenase/tryptophan 5-monooxygenase activation protein, gamma polypeptide (14-3-3 gamma)	Odin Transfection Only
YWHAZ	tyrosine 3/tryptophan 5 -monooxygenase activation protein, zeta polypeptide (14-3-3 theta)	Odin Transfection Only
YWHAB	tyrosine 3-monooxygenase/tryptophan 5-monooxygenase activation protein, beta polypeptide (14-3-3 beta)	Odin Transfection Only
YWHAH	tyrosine 3/tryptophan 5 -monooxygenase activation protein, eta polypeptide (14-3-3 eta)	Odin Transfection Only
YWHAE	tyrosine 3/tryptophan 5 -monooxygenase activation protein, epsilon polypeptide (14-3-3 epsilon)	Odin Transfection Only
SFN	stratifin (14-3-3 sigma)	Odin Transfection Only
SH3KBP1	SH3-domain kinase binding protein 1	Odin Transfection Only
CD2AP	CD2-associated protein (CMS)	Odin Transfection Only
RASAL2	RAS protein activator like 2 isoform 2	Odin Transfection Only
YWHAQ	tyrosine 3/tryptophan 5 -monooxygenase activation protein, theta polypeptide (14-3-3 theta)	Odin Transfection Only
CAPZB	F-actin capping protein beta subunit	Odin Transfection Only
DAB2IP	DAB2 interacting protein isoform 1	Odin Transfection Only
TLN2	talin 2	Odin Transfection Only
GART	phosphoribosylglycinamide formyltransferase, phosphoribosylglycinamide synthetase, phosphoribosylaminoimidazole synthetase	Odin Transfection Only
VAPA	vesicle-associated membrane protein-associated protein A isoform 1	Odin Transfection Only
ARHGAP10	Rho GTPase activating protein 10	Odin Transfection Only
HSPA9B	heat shock 70kDa protein 9B precursor	Odin Transfection Only
UACA	uveal autoantigen with coiled-coil domains and ankyrin repeats isoform 1	Odin Transfection Only
HSPA6	heat shock 70kDa protein 6 (HSP70B')	4.23
HSPA1B	heat shock 70kDa protein 1B	4.02
PRDX1	peroxiredoxin 1	3.59
PPP2R2A	alpha isoform of regulatory subunit B55, protein phosphatase 2	3.55
HSPA8	heat shock 70kDa protein 8 isoform 1	3.47
ARL1	ADP-ribosylation factor-like 1	3.15
TUBA1B	tubulin, alpha, ubiquitous	3.11
PHGDH	phosphoglycerate dehydrogenase	3.06
TUBB	tubulin, beta polypeptide	3.04
PGAM5	Bcl-XL-binding protein v68	3
EEF1A1	eukaryotic translation elongation factor 1 alpha 1	2.84
TMEM33	transmembrane protein 33	2.82
PPP2R1A	alpha isoform of regulatory subunit A, protein phosphatase 2	2.8
PRNPIP	prion protein interacting protein	2.74
PRDX2	peroxiredoxin 2 isoform a	2.7
SLC25A6	solute carrier family 25, member A6	2.6
PRDX4	thioredoxin peroxidase (peroxiredoxin 4)	2.59
EEF2	eukaryotic translation elongation factor 2 [Homo sapiens]	2.58